invitrogen™

pSecTag2/Hygro A, B, and C

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User Manual

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Kit Contents and Storage

Kit Contents	Each kit contains 20 µg each of pSecTag2/Hygro A, B, and C and pSecTag2/Hygro/PSA. Each vector is supplied at a concentration of 0.5 µg/µl in 40 µl of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
Shipping and Storage	Plasmids are shipped on ice and should be stored at –20°C.
Product Qualification	The Certificate of Analysis (CofA) provides detailed quality control information for each product. The CofA is available on our website at www.invitrogen.com/cofa, and is searchable by product lot number, which is printed on each box.

Additional Products

Kit Contents

The following products are available separately from Invitrogen. To order, visit our website at www.invitrogen.com or contact Technical Support (see page 13).

Product	Amount	Catalog number
PureLink™ HiPure Plasmid MiniPrep Kits	25 preps 100 preps	K2100-02 K2100-03
PureLink™ HiPure Plasmid MidiPrep Kits	25 preps 50 preps	K2100-04 K2100-05
ChargeSwitch®-Pro Plasmid Miniprep Kit	50 preps 250 preps	CS30050 CS30250
Anti-*myc* Antibody	25 westerns	R950-25
Anti-His (C-term) Antibody	25 westerns	R930-25
ProBond™ Purification System	6 purifications	K850-01
ProBond™ Metal-Binding Resin	50 ml 150 ml	R801-01 R801-15
Purification Columns (10-ml polypropylene columns)	50	R640-50

Introduction

Overview

Introduction

pSecTag2/Hygro A, B, and C are 5.7 kb expression vectors designed for high-level expression and secretion in mammalian hosts. The pSecTag2/Hygro vectors are identical to the pSecTag2 vectors except that the Zeocin™ resistance gene is replaced with the hygromycin B resistance gene (Gritz and Davies, 1983) for selection in mammalian cells. Like pSecTag2, pSecTag2/Hygro contains the gene encoding β-lactamase for bacterial selection on ampicillin.

Proteins expressed from pSecTag2/Hygro are fused at the N-terminus to the murine Ig kappa chain leader sequence for protein secretion and at the C-terminus to a peptide containing the *c-myc* epitope and six tandem histidine residues for detection and purification.

The pSecTag2/Hygro vector is supplied in three different versions (A, B, and C) to facilitate correct in-frame fusion with the Ig kappa chain leader sequence.

For more information on the pSecTag2/Hygro vector, see page 2.

To get started with cloning into pSecTag2/Hygro, see page 4.

E. coli Strain

We recommend that you propagate pSecTag2/Hygro A, B and C in *E. coli* strains that are recombination deficient (*rec*A) and endonuclease A deficient (*end*A) such as TOP10F´, DH5αF´, and INVαF´.

For your convenience, TOP10F´ is available as chemically competent or electrocompetent cells from Invitrogen.

Item	Quantity	Catalog no.
Electrocomp™ TOP10F´	5 × 80 µl	C665-55
One Shot® TOP10F´ (chemically competent cells)	21 × 50 µl	C3030-03

pSecTag2/Hygro Vector

Features of pSecTag2/Hygro

pSecTag2/Hygro A (5745 bp), pSecTag2/Hygro B (5749 bp), and pSecTag2/Hygro C (5753 bp) contain the following elements. All features have been functionally tested.

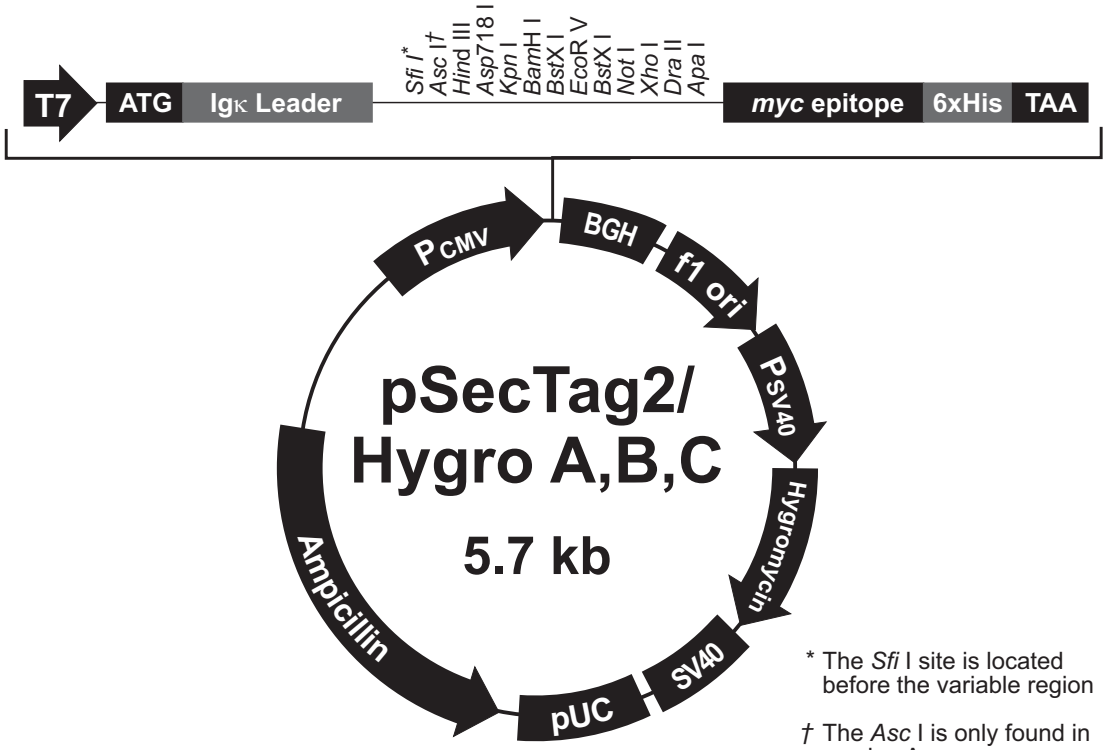
Feature	Benefit
Human cytomegalovirus (CMV) immediate-early promoter/enhancer	Allows efficient, high-level expression of your recombinant protein (Andersson *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987)
T7 promoter/priming site	Allows for *in vitro* transcription in the sense orientation and sequencing through the insert
ATG initiation codon	Allows initiation of translation of the pSecTag2/Hygro fusion protein
Murine Ig κ-chain leader sequence	Allows secretion of the fusion protein (Coloma *et al.*, 1992)
Multiple cloning site	Allows insertion of your gene and facilitates cloning
c-myc epitope (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu)	Allows detection of pSecTag2/Hygro fusion protein with the Anti-*myc* Antibody (Catalog no. R950-25) (Evans *et al.*, 1985)
Polyhistidine tag	For high affinity binding to Ni^{2+}-chelating resin (i.e. ProBond™) and easy purification In addition, it allows detection of pSecTag2/Hygro fusion proteins with the Anti-His(C-term) Antibody (Catalog no. R930-25) (Lindner *et al.*, 1997)
BGH reverse priming site	Allows sequencing through the insert
Bovine growth hormone (BGH) polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA (Goodwin and Rottman, 1992)
f1 origin	Allows rescue of single-stranded DNA
SV40 early promoter and origin	Allows efficient, high-level expression of the hygromycin resistance gene and episomal replication in cells expressing the SV40 large T antigen
Hygromycin resistance gene (Hygromycin B-phosphotransferase)	Selection of stable transfectants in mammalian cells (Gritz and Davies, 1983; Palmer *et al.*, 1987)
SV40 polyadenylation signal	Efficient transcription termination and polyadenylation of mRNA
pUC origin	High-copy number replication and growth in *E. coli*
Ampicillin resistance gene (β-lactamase)	Selection in *E. coli*

continued on next page

pSecTag2/Hygro Vector, continued

Map of pSecTag2/Hygro

The figure below shows where the features of pSecTag2/Hygro are located in the vector. The sequences for pSecTag2/Hygro A, B, and C are available for downloading from our Web site (www.invitrogen.com) or from Technical Service (page 13). Details of the multiple cloning sites for all three vectors, including the variable region that determines the reading frame, are shown on pages 5-7.



* The *Sfi* I site is located before the variable region

† The *Asc* I is only found in version A.

Comments for pSecTag2/Hygro A
5745 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Murine Ig kappa-chain V-J2-C signal peptide: bases 905-967
Multiple cloning site: bases 970-1081
c-myc epitope: bases 1082-1111
Polyhistidine tag: bases 1127-1144
BGH reverse priming site: bases 1167-1184
BGH polyadenylation site: bases 1166-1380
f1 origin: bases 1443-1856
SV40 promoter and origin: bases 1924-2245
Hygromycin B phosphotransferase ORF (Hyg^R): bases 2263-3288
SV40 polyadenylation site: bases 3418-3547
pUC origin: bases 3931-4604
β-lactamase ORF (Amp^R): bases 4749-5609

Methods

Cloning into pSecTag2/Hygro

Introduction

This section contains information on cloning your insert into the pSecTag2/Hygro vectors. Details of the multiple cloning sites are found on pages 5-7. Brief information on transforming into *E. coli* is located below.

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, see *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994)

Maintenance of pSecTag2/Hygro

To propagate and maintain pSecTag2/Hygro A, B, and C, we recommend that you transform the plasmids into *E. coli* and prepare glycerol stocks for long-term storage as described below:

1. Use the supplied 0.5 µg/µl stock solution (in TE, pH 8.0) to transform a *rec*A, *end*A *E. coli* strain like INVαF´, TOP10F´, DH5αF´, or equivalent.
2. Select transformants on LB plates containing 50–100 µg/ml ampicillin.
3. Analyze transformants for the appropriate plasmid and prepare glycerol stocks by mixing 0.85 ml of an overnight culture with 0.15 ml of sterile glycerol. Transfer the resulting solution to a cryovial and store at –80°C.

Cloning into the pSecTag2/Hygro Vectors

pSecTag2/Hygro A, B, and C vectors are fusion vectors requiring that you clone your gene of interest in frame with the initiation ATG of the N-terminal Ig kappa chain leader sequence and/or the C-terminal *myc* epitope/polyhistidine tag. Three versions of this vector are provided to facilitate cloning. For proper expression, first determine which restriction sites are appropriate for ligation and then which vector will preserve the reading frame at **BOTH** the 5´ and the 3´ ends. It may be necessary to PCR your gene product to create a fragment with the appropriate restriction sites to clone in frame at both ends. Carefully inspect your gene and the multiple cloning site of each vector before cloning your gene of interest. Be sure to remove the stop codon in your gene if you wish to express your protein with the C-terminal tag. See pages 5-7 for details of the multiple cloning sites.

Note

If you wish to express and secrete your protein without the C-terminal tag, include a termination codon in your gene of interest. Note that you will be unable to detect the fusion protein with the Anti-*myc* Antibody or Anti-His (C-term) Antibody or purify it using nickel-chelating resin (i.e. ProBond™).

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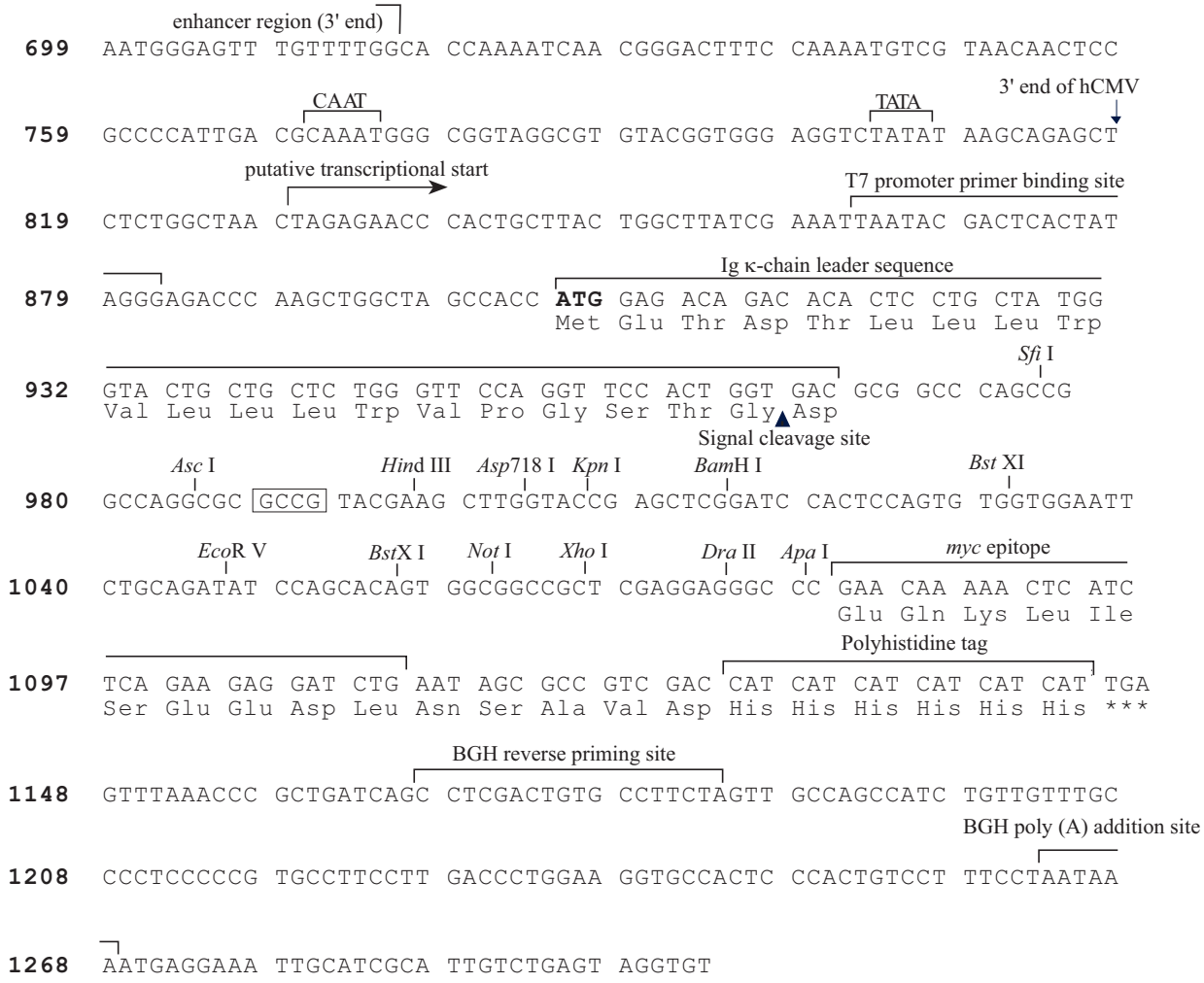
Cloning into pSecTag2/Hygro, continued

Multiple Cloning Site of pSecTag2/ Hygro A

Below is the multiple cloning site for pSecTag2/Hygro A. Restriction sites are labeled to indicate the cleavage site. The variable region is the boxed region located after the Ig kappa chain leader sequence. The multiple cloning site has been confirmed by sequencing and functional testing.


enhancer region (3' end)
699 AATGGGAGTT TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG TAACAACTCC
CAAT
TATA
3' end of hCMV
759 GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT AAGCAGAGCT
putative transcriptional start
T7 promoter primer binding site
819 CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC GACTCACTAT
Ig κ-chain leader sequence
879 AGGGAGACCC AAGCTGGCTA GCCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG
Met Glu Thr Asp Thr Leu Leu Leu Trp
Sfi I
932 GTA CTG CTG CTC TGG GTT CCA GGT TCC ACT GGT GAC GCG GCC CAGCCG
Val Leu Leu Leu Trp Val Pro Gly Ser Thr Gly Asp
Signal cleavage site
Asc I
Hind III
Asp718 I
Kpn I
BamH I
Bst XI
980 GCCAGGCGC GCCG TACGAAG CTTGGTACCG AGCTCGGATC CACTCCAGTG TGGTGGAATT
EcoR V
BstX I
Not I
Xho I
Dra II
Apa I
myc epitope
1040 CTGCAGATAT CCAGCACAGT GGCGGCCGCT CGAGGAGGGC CC GAA CAA AAA CTC ATC
Glu Gln Lys Leu Ile
Polyhistidine tag
1097 TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA
Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His His His His His His ***
BGH reverse priming site
1148 GTTTAAACCC GCTGATCAGC CTCGACTGTG CCTTCTAGTT GCCAGCCATC TGTTGTTTGC
BGH poly (A) addition site
1208 CCCTCCCCCG TGCCTTCCTT GACCCTGGAA GGTGCCACTC CCACTGTCCT TTCCTAATAA
1268 AATGAGGAAA TTGCATCGCA TTGTCTGAGT AGGTGT

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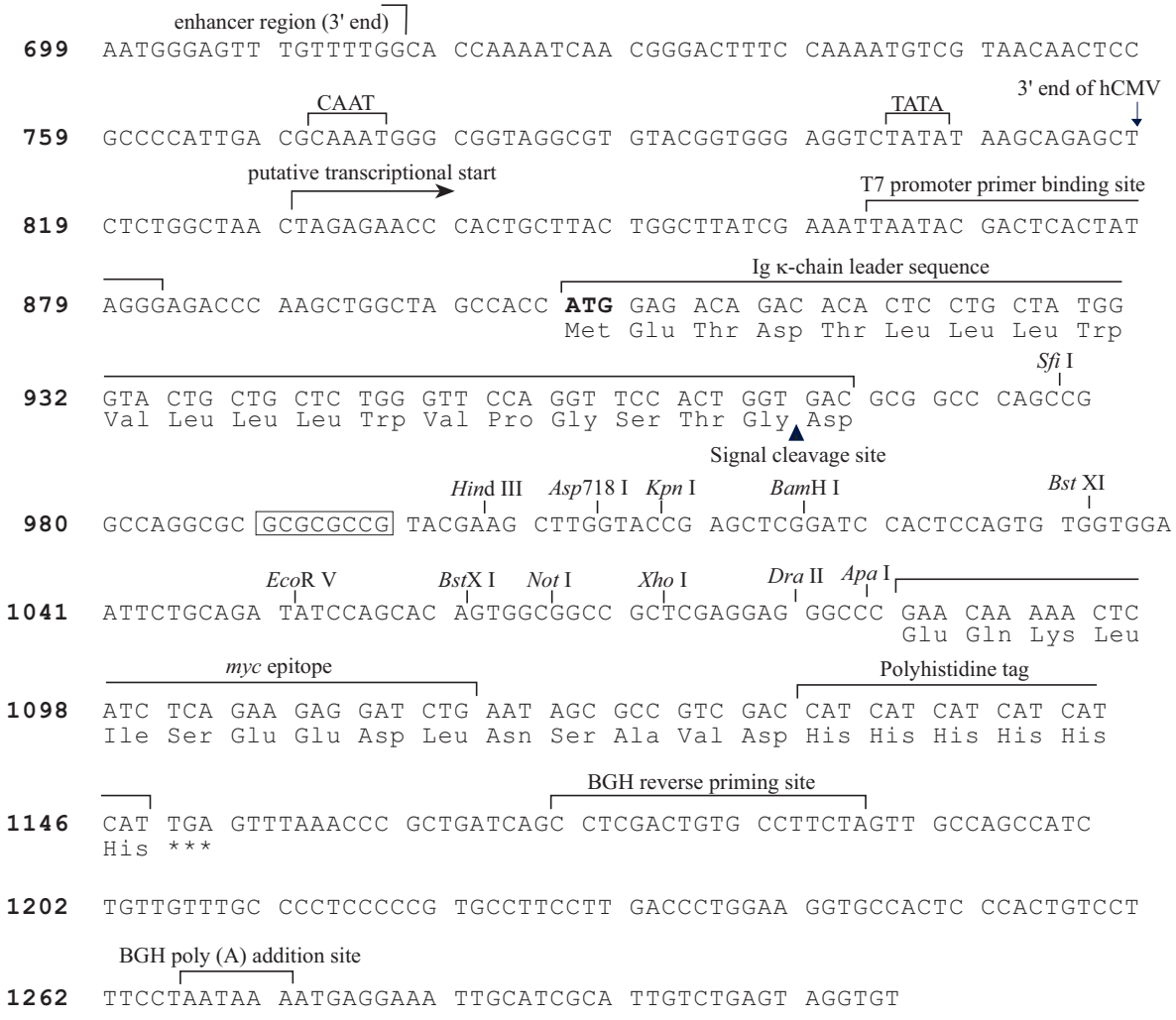
Cloning into pSecTag2/Hygro, continued

Multiple Cloning Site of pSecTag2/ Hygro B

Below is the multiple cloning site for pSecTag2/Hygro B. Restriction sites are labeled to indicate the cleavage site. The variable region is the boxed region located after the Ig kappa chain leader sequence. The multiple cloning site has been confirmed by sequencing and functional testing.


enhancer region (3' end)
699 AATGGGAGTT TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG TAACAACTCC
CAAT
TATA
3' end of hCMV
759 GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT AAGCAGAGCT
putative transcriptional start
T7 promoter primer binding site
819 CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC GACTCACTAT
Ig κ-chain leader sequence
879 AGGGAGACCC AAGCTGGCTA GCCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG
Met Glu Thr Asp Thr Leu Leu Leu Trp
Sfi I
932 GTA CTG CTG CTC TGG GTT CCA GGT TCC ACT GGT GAC GCG GCC CAGCCG
Val Leu Leu Leu Trp Val Pro Gly Ser Thr Gly Asp
Signal cleavage site
Hind III
Asp718 I
Kpn I
BamH I
Bst XI
980 GCCAGGCGC GCGCGCCG TACGAAG CTTGGTACCG AGCTCGGATC CACTCCAGTG TGGTGGA
EcoR V
BstX I
Not I
Xho I
Dra II
Apa I
1041 ATTCTGCAGA TATCCAGCAC AGTGGCGGCC GCTCGAGGAG GGCCC GAA CAA AAA CTC
Glu Gln Lys Leu
myc epitope
Polyhistidine tag
1098 ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT
Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His His His His His
BGH reverse priming site
1146 CAT TGA GTTTAAACCC GCTGATCAGC CTCGACTGTG CCTTCTAGTT GCCAGCCATC
His ***
1202 TGTTGTTTGC CCCTCCCCCG TGCCTTCCTT GACCCTGGAA GGTGCCACTC CCACTGTCCT
BGH poly (A) addition site
1262 TTCCTAATAA AATGAGGAAA TTGCATCGCA TTGTCTGAGT AGGTGT

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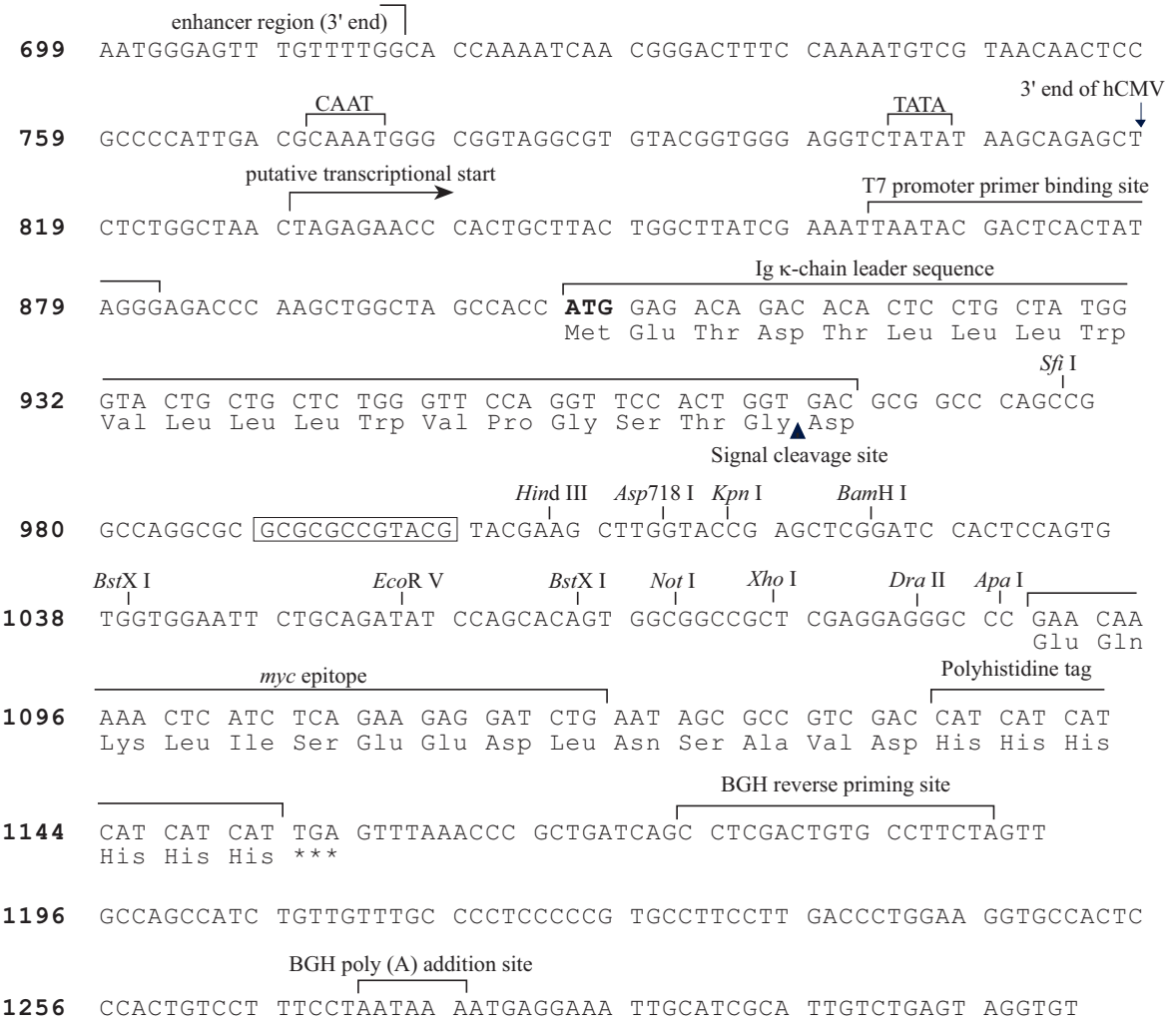
Cloning into pSecTag2/Hygro, continued

Multiple Cloning Site of pSecTag2/ Hygro C

Below is the multiple cloning site for pSecTag2/Hygro C. Restriction sites are labeled to indicate the cleavage site. The variable region is the boxed region located after the Ig kappa chain leader sequence. The multiple cloning site has been confirmed by sequencing and functional testing.


enhancer region (3' end)
699 AATGGGAGTT TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG TAACAACTCC
CAAT
TATA
3' end of hCMV
759 GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT AAGCAGAGCT
putative transcriptional start
T7 promoter primer binding site
819 CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC GACTCACTAT
Ig κ-chain leader sequence
879 AGGGAGACCC AAGCTGGCTA GCCACC ATG GAG ACA GAC ACA CTC CTG CTA TGG
Met Glu Thr Asp Thr Leu Leu Leu Trp
Sfi I
932 GTA CTG CTG CTC TGG GTT CCA GGT TCC ACT GGT GAC GCG GCC CAGCCG
Val Leu Leu Leu Trp Val Pro Gly Ser Thr Gly Asp
Signal cleavage site
Hind III Asp718 I Kpn I BamH I
980 GCCAGGCGC GCGCGCCGTACG TACGAAG CTTGGTACCG AGCTCGGATC CACTCCAGTG
BstX I EcoR V BstX I Not I Xho I Dra II Apa I
1038 TGGTGGAATT CTGCAGATAT CCAGCACAGT GGCGGCCGCT CGAGGAGGGC CC GAA CAA
Glu Gln
myc epitope
Polyhistidine tag
1096 AAA CTC ATC TCA GAA GAG GAT CTG AAT AGC GCC GTC GAC CAT CAT CAT
Lys Leu Ile Ser Glu Glu Asp Leu Asn Ser Ala Val Asp His His His
BGH reverse priming site
1144 CAT CAT CAT TGA GTTTAAACCC GCTGATCAGC CTCGACTGTG CCTTCTAGTT
His His His ***
1196 GCCAGCCATC TGTTGTTTGC CCCTCCCCCG TGCCTTCCTT GACCCTGGAA GGTGCCACTC
BGH poly (A) addition site
1256 CCACTGTCCT TTCCTAATAA AATGAGGAAA TTGCATCGCA TTGTCTGAGT AGGTGT

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Transformation into *E. coli*

Introduction

At this point you should have ligation mixtures that are ready to be transformed into competent *E. coli*. The following guidelines and recommendations are provided for your convenience. If you need more details about the techniques discussed, refer to the general molecular biology references in the **Reference** section.

E. coli Transformation

Transform your ligation mixtures into a competent *rec*A, *end*A *E. coli* strain (e.g. INVαF´, TOP10F´, DH5α) and select on LB plates containing 50-100 µg/ml ampicillin. Select 10-20 clones and analyze for the presence and orientation of your insert.

We recommend that you sequence your construct with the T7 forward and BGH reverse primer binding sites to confirm that your gene is correctly fused to the Ig kappa chain leader sequence at the N-terminal and the C-terminal tag. Refer to diagrams on pages 5-7 for sequence and location of primer binding sites.

For your convenience, Invitrogen offers a custom primer synthesis service. For more information, refer to our website (www.invitrogen.com) or contact Technical Support (page 13).

Plasmid Preparation

Once you have confirmed that your gene is in the correct reading frame, prepare plasmid DNA for transfection. Plasmid DNA for transfection into eukaryotic cells must be very clean and free from phenol and sodium chloride. Contaminants will kill the cells and salt will interfere with lipid complexing, decreasing transfection efficiency. We recommend isolating DNA using the PureLink™ HiPure Plasmid MiniPrep or Plasmid MidiPrep kits, or the ChargeSwitch®-Pro Plasmid Miniprep Kit (see page vi, **Additional Products**).

Transfection into Mammalian Cells

Methods of Transfection

For established cell lines (*e.g.,* HeLa), consult original references or the supplier of your cell line for the optimal method of transfection. It is recommended that you follow exactly the protocol for your cell line. Pay particular attention to medium requirements, when to pass the cells, and at what dilution to split the cells. Further information is provided in *Current Protocols in Molecular Biology*.

Hygromycin B Selection Guidelines

If you wish to create stable cell lines expressing your gene of interest you will need to select using hygromycin B. Use hygromycin B (527.5 MW) as follows:

- Test varying concentrations of hygromycin on your cell line to determine the concentration that kills your cells (kill curve). Cells differ in their susceptibility to hygromycin.
- Prepare complete medium supplemented with 100 to 1000 µg/ml hygromycin B. Generally, concentrations that kill mammalian cells are in the 150 to 400 µg/ml range.
- Calculate concentration based on the amount of active drug (check the lot label).

  Cells will divide once or twice in the presence of lethal doses of hygromycin, so the effects of the drug take several days to become apparent. Complete inhibition of cell growth can take 2 to 3 weeks of growth in selective medium. Selection and expansion of clones will take additional time.

Hygromycin B Activity

Hygromycin B (Catalog no. 10687-010) is an aminocyclitol that inhibits protein synthesis by disrupting translocation and promoting mistranslation. It is used for selection of stable mammalian cell lines. The resistance gene encodes hygromycin B-phosphotransferase, which detoxifies hygromycin B by phosphorylation.

Expression and Purification

Introduction

Expression of your recombinant protein can be detected using an antibody to the *c-myc* epitope encoded in the C-terminal fusion peptide. In addition, the metal binding domain allows simple, one-step purification of your recombinant protein by Immobilized Metal Affinity Chromatography (IMAC) using a nickel-chelating resin. (*i.e.,* ProBond™). See **Additional Products** on page vi for detection and purification products from Invitrogen.

Detection of Secreted Protein from Medium

The medium in which transfected cells are grown can be analyzed for secreted, recombinant protein by functional assay or western blot analysis. It may be necessary to perform a time course analysis to determine the optimal time for expression of your recombinant protein. If you do not have an antibody to your particular protein, you can use the Anti-*myc* Antibody to detect your protein.

A positive control vector, pSecTag2/Hygro/PSA, is included to test for expression and secretion in your particular cell line. Prostate-specific antigen (PSA) is fused to the *c-myc* epitope and the polyhistidine tag. The resulting fusion protein is ~33 kDa which includes the N-terminal secretion signal. When the secretion signal is cleaved off, the size of the fusion protein should decrease by 2.4 kDa. **Note**: There are glycosylation sites in PSA. The secreted fusion protein expressed in human breast carcinoma cells migrates at ~45 kDa.

Purification of Secreted Recombinant Protein

To purify secreted, recombinant protein from the medium, follow the manufacturer's instructions for the nickel-chelating resin that you are using. Start with about 3 to 5 ml of medium and load onto 1 to 2 ml of resin. Scale up or down depending on the level of expression.

Analyzing Cells for Recombinant Protein

If you do not detect any secreted protein in the medium, use the procedure below to check cells for production of recombinant protein. You will need 5×10^6 to 1×10^7 cells for purification on a 2 ml ProBond™ column (see ProBond™ Protein Purification manual).

1. Seed cells in either five T-75 flasks or 2 to 3 T-175 flasks.
2. Grow the cells in selective medium until they are 80-90% confluent.
3. Harvest the cells by treating with trypsin-EDTA for 2 to 5 minutes or by scraping the cells in PBS.
4. Inactivate the trypsin by diluting with complete medium. Transfer the cells to a sterile conical tube.
5. Centrifuge the cells at 1500 rpm for 5 minutes. You may wish to wash the cells one time with PBS prior to lysis. The cells may be lysed immediately or frozen in liquid nitrogen and stored at -80°C until needed.

Lysis of Cells

If you are using ProBond™ resin, refer to the ProBond™ Protein Purification manual for details about sample preparation for chromatography.

If you are using other resin, refer to the manufacturer's instruction for recommendations on sample preparation.

Appendix

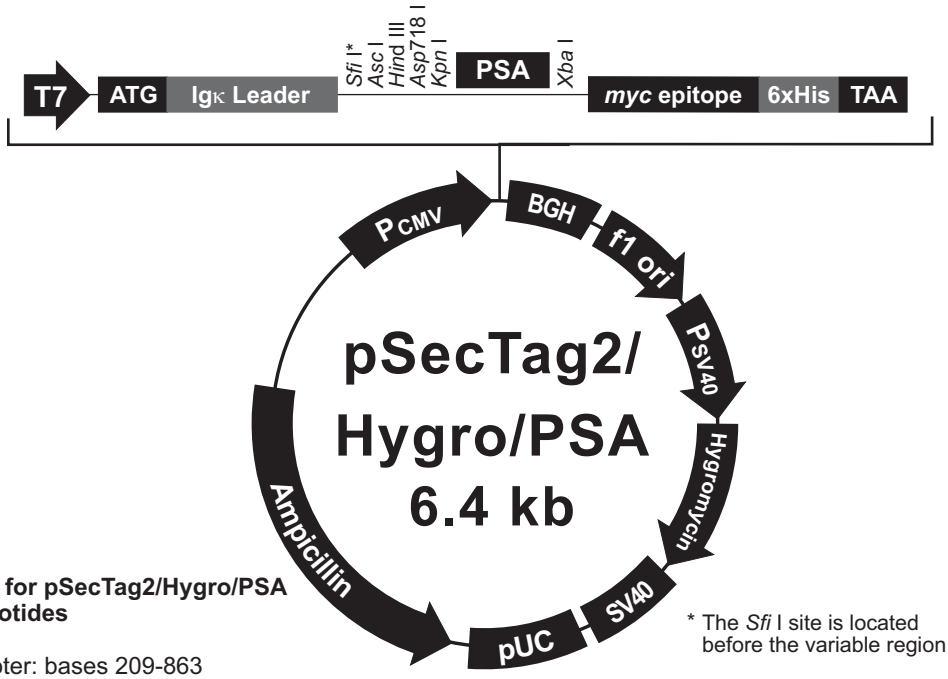
pSecTag2/Hygro/PSA

Description

pSecTag2/Hygro/PSA is a 6408 bp positive control vector expressing and secreting the prostate-specific antigen (PSA) fused to the *c-myc* epitope and the polyhistidine tag. The vector was constructed by amplifying the PSA gene and cloning it into pCR®II. The fragment of DNA containing the PSA gene was excised using *Kpn* I and *Apa* I and cloned into *Kpn* I/*Apa* I digested pSecTag2/Hygro A.

Map

The figure below shows the features of pSecTag2/Hygro/PSA. The complete nucleotide sequence for pSecTag2/Hygro/PSA is available for downloading from our Web site (www.invitrogen.com) or from Technical Service (page 13).



* The *Sfi* I site is located before the variable region

Comments for pSecTag2/Hygro/PSA
6408 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Murine Ig kappa-chain V-J2-C signal peptide: bases 905-967
PSA gene: bases 1042-1732
c-myc epitope: bases 1745-1777
Polyhistidine tag: bases 1790-1807
BGH reverse priming site: bases 1830-1847
BGH polyadenylation sequence: bases 1829-2043
f1 origin: bases 1954-2367
SV40 promoter and origin: bases 2587-2908
Hygromycin B phosphotransferase ORF (Hyg^R): bases 2926-3951
SV40 polyadenylation site: bases 4144-4210
pUC origin: bases 4594-5267
β-lactamase ORF (Amp^R): bases 5412-6272

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

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MSDS

Material Safety Data Sheets (MSDSs) are available on our website at www.invitrogen.com/msds.

Certificate of Analysis

The Certificate of Analysis (CofA) provides detailed quality control information for each product. The CofA is available on our website at www.invitrogen.com/cofa, and is searchable by product lot number, which is printed on each box.

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Purchaser Notification

Limited Use Label License No. 22: Vectors and Clones Encoding Histidine Hexamer

This product is licensed under U.S. Patent Nos. 5,284,933 and 5,310,663 and foreign equivalents from Hoffmann-LaRoche, Inc., Nutley, NJ and/or Hoffmann-LaRoche Ltd., Basel, Switzerland and is provided only for use in research. Information about licenses for commercial use is available from QIAGEN GmbH, Max-Volmer-Str. 4, D-40724 Hilden, Germany.

References

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